Effect of moderate hypoxia on cultured vascular smooth muscle cells

Honors Research Project

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Introduction

Arteries carry oxygen to tissues and take carbon dioxide away. The distribution of blood flow within the body is determined by the degree of contraction of the vascular smooth muscle. When the muscle contracts, less blood flows to the region. When the muscle relaxes, blood flow increases (5). Oxygen delivery must be sufficient to meet the metabolic needs of the tissues in the blood vessels as well as the areas supplied (fig. 1).

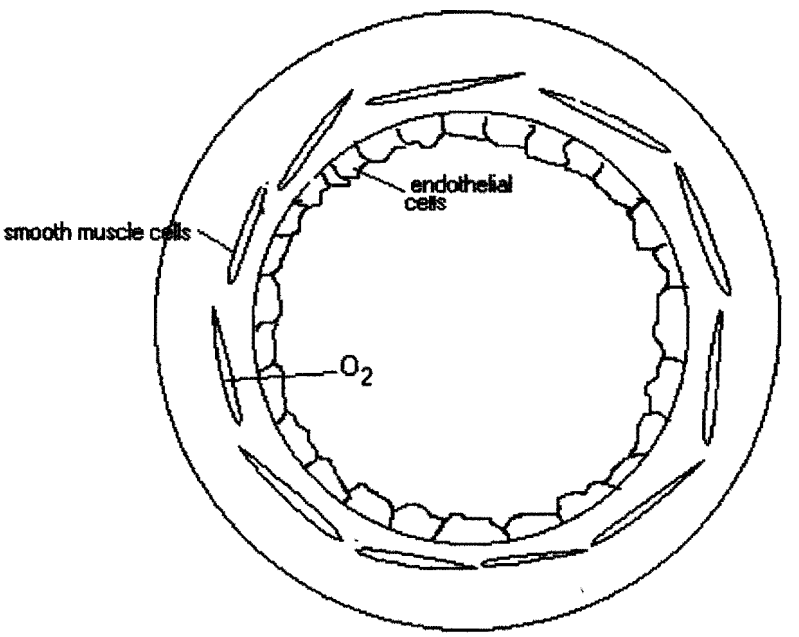

Fig. 1. Arterial blood vessel. Oxygen must pass from the blood in the vessel to the muscle cells in the walls of the vessel.

In general, the degree of muscle contraction is proportional to the concentration of free calcium ($[Ca^{++}]$) in the myoplasm, which is controlled by a variety of mechanisms. When $[Ca^{++}]$ increases, calcium binds to calmodulin. The calcium-calmodulin complex combines with myosin light chain kinase (MLCK), activating it and inducing phosphorylation of the 20kD light chain of myosin (LC_{20}). Phosphorylation of LC_{20} allows the myosin to interact with actin, leading to contraction (4 and 8) (fig.2).

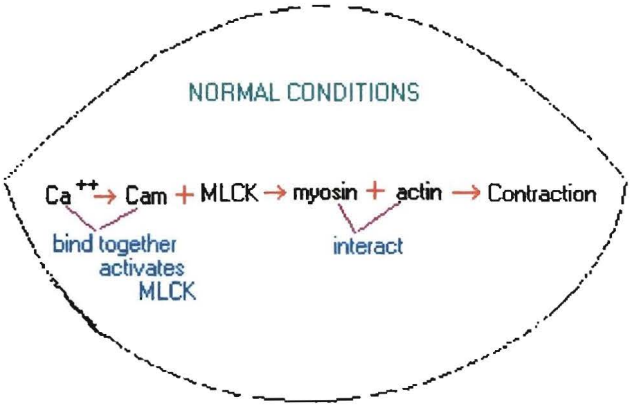

Fig. 2. Smooth muscle cell. Under normal conditions Ca^{++} and calmodulin bind activating the myosin light chain kinase, in turn myosin and actin interact and the muscle contracts.

However, not all changes in the contractile state of smooth muscle can be explained by changes in $[Ca^{++}]$, leading investigators to speculate on alternate regulatory mechanisms.

Problem Identification and Justification

It is well known that traumatic states such as shock or lack of oxygen reduce the ability of smooth muscle to contract; however the mechanisms responsible for this finding are not clear. Drs. Brooks and Olson have developed a novel hypothesis to explain why vascular smooth muscle becomes unresponsive to contractile stimuli when the muscle becomes hypoxic. They have proposed that hypoxia alters signal transduction in vascular smooth muscle, which changes the relationship between $[Ca^{++}]$ and contraction. They have recently shown that exposing excised vascular smooth muscle to one hour of moderate hypoxia both reduces the contractile force generated in response to either KCl or norepinephrine, and increases the quantity of Heat Shock Protein 90 (**HSP90**) within the smooth muscle cells (1). Based on a review of the literature, Drs.

Brooks and Olson have identified several mechanisms by which increases in intracellular HSP90 could result in the observed reduction in contractile force. This research tested one of those mechanisms in cultured smooth muscle cells.

Specifically, we propose that increases in HSP90 reduce vascular contractility by decreasing MAP Kinase activity. MAP Kinase is activated by agonists such as KCl. Caldesmon, a smooth muscle protein, is a known substrate for MAP Kinase. Caldesmon normally inhibits the actin-myosin ATPase, the enzyme responsible for actin-myosin interaction, which is the final step in muscle contraction. This inhibition is released when caldesmon is phosphorylated by MAP Kinase. A reduction in MAP Kinase activity would maintain caldesmon in its unphosphorylated, and therefore inhibitory, form, which could explain the reduction in contractile force observed in hypoxic smooth muscle.

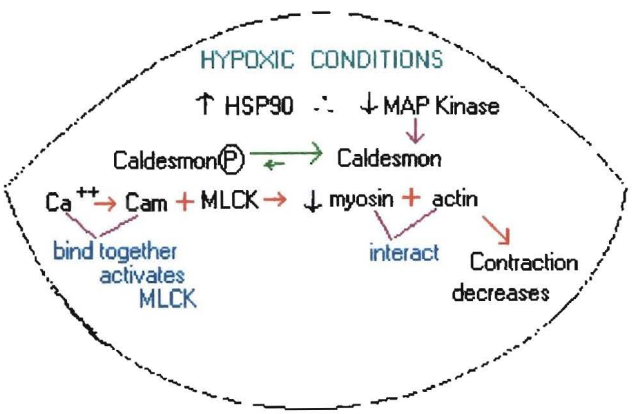

Fig. 3. Smooth muscle cell. Under hypoxic conditions HSP90 increases and MAP Kinase decreases, caldesmon is dephosphorylated and the contraction decreases.

Hypothesis and Objectives

After evaluation of the known reactions in a smooth muscle cell a hypothesis concerning the unknown reactions was produced. Exposing cultured vascular smooth

muscle cells to one hour of moderate hypoxia will increase HSP90, leading to a decrease in MAP Kinase activity. Two specific objectives were created to test this hypothesis.

1) - Determine whether cultured vascular smooth muscle cells respond to one hour of moderate hypoxia like excised vascular tissue, by increasing HSP90.

Cultured cells were exposed to the same conditions as previously used to test excised rat aorta and results were compared.

2) - Determine the effect of one hour of moderate hypoxia on MAP Kinase activity in cultured vascular smooth muscle cells.

Cultured cells, normal and hypoxic, were tested with a MAP Kinase assay to determine MAP Kinase activity.

Procedures and Methods

Technique Development

In the original research performed by Almgren and Olson it was shown that HSP90 concentration increased under hypoxic conditions in the aortic tissue excised from rats (2). The first objective of this research was to show that cultured cells of rat thoracic smooth muscle reacted in the same manner. Reproducing the results in cultured cells would allow additional experiments regarding the timing of the HSP90 induction to be performed in a cell system that is likely more homogeneous and well controlled than freshly excised tissue. An immortalized line of rat thoracic vascular smooth muscle cells (A10; American *Type Culture* Collection #CRL 1476) was cultured. The A10 cells are derived from the thoracic aorta of embryonic rat. It has been shown that they possess many of the same characteristics of smooth muscle (6). The cells were grown in tissue culture plates using Dulbecco's modified eagle's medium (**DMEM**) supplemented with 10% non heat-inactivated fetal bovine serum in a 37°C cell culture incubator. The tests used plates that were at least 85% confluent with cells and between passages 20 to 30.

Antibody Titration

A preliminary dot blot was run with the goal of accurately detecting the proteins present and to find which concentration of secondary antibody and which method of protein detection was most useful. The plates were rinsed with sterile phosphate buffered saline (**PBS**). 200 µl of Tris/NaCl/EDTA homogenization buffer was added to each of the plates, which were scraped, the cells were crushed in Kontes grinders, thus the cells were lysed. The mixture was spun at 14,000 x G for 3 minutes at 4°C. The protein concentration was determined on the supernatant with a BCA test and the samples were spotted in duplicate on the blot in 1X, 1/5, 1/10, and 1/100 concentrations. The blot was divided, one set was detected with colorimetric immunoblotting using 1/3000, 1/1000 and 1/500 primary antibody dilutions and the other by chemiluminescent immunoblotting with the same dilutions. First the blots were blocked with 2% nonfat dry milk in TBS then the primary antibody was added which binds to the HSP90 protein. The differing detection methods require secondary antibodies conjugated with different enzymes as this is what is detected. The colorimetric detection uses goat anti mouse IgG conjugated with alkaline phosphate, and the chemiluminescent requires a sheep anti mouse IgG conjugated with horseradish peroxidase.

Homogenization buffer and Protein assays

The buffer used in the excised rat aorta experiment was a Tris/NaCl/EDTA buffer. A different homogenization buffer, containing phosphotase and protease inhibitors, was needed for the MAP Kinase assay, which necessitated a different protein assay. The MAP Kinase buffer contained triton and 2 mercaptoethanol, which interfered with the BCA protein analysis. In these experiments the protein concentration was determined using a Bradford protein assay.

HSP90 detection by immunoblotting (Western)

The plated cells were rinsed with PBS as above. 200µl of MAP Kinase homogenization buffer was added and the cells were lysed as above. The protein was extracted and concentration was determined via a Bradford protein assay (3). It was found that a higher concentration of protein was needed in the extract to have a high enough quantity in the 20-30µl sample aliquot loaded into the gel. On later plates used the PBS was suctioned off before the homogenization buffer was added to reduce the dilution of the protein. Cell extracts containing 25 to 50 µg of protein were combined with Laemmli sample buffer and separated by SDS-PAGE (polyacrylamide gel electrophoresis) on 7.5% gels. On many gels a standard of pure HSP90 protein (SPP770 StressGen, Inc.) and a rainbow marker were loaded for comparison. After an hour of electric current, the protein was then tank-transferred to nitrocellulose paper. Detection of the protein was preceded by washing the paper with Tris-buffered saline containing 2% dry milk. Primary and secondary antibodies were used at a 1/500 dilution. Proteins were then visualized using a colorimetric detection method (BCIP/NBT).

Hypoxia effects

Once HSP90 was detectable the cells were divided into a control group and test group. The test cells were exposed to moderate hypoxia for an hour enclosed in a hypoxic chamber (fig. 4), kept in a 37°C room.

Fig. 4. Hypoxia chamber. Plates of cells were incubated for an hour with hypoxic gas in this chamber.

Prior to testing, hypoxic gas (2.98% O_2 5.01% CO_2, balance N_2) was bubbled through fresh media in order to precondition the media. Control media was equilibrated by placing the amount needed in a 37°C incubator that was gassed continuously with 5% CO_2 in air. The preconditioned media was added to the plates and the cells were left at 37°C for the time remaining. Control cells were left in the environment in which the cells had grown, the 37°C incubator. After exposure to the hypoxic and normoxic conditions the media was removed and the plates were treated the same as above. The plates were kept on ice to reduce cellular metabolism. Once again, a Bradford protein assay was done to determine protein concentration. The samples were diluted to standardize the concentration of protein between the test and control samples. Electrophoresis gels were run and the proteins were transferred to nitrocellulose paper, blocked and immunoblotted. HSP90 was then made visible on the paper by colorimetric detection (fig. 5).

MAP Kinase Assay by immunoprecipitation

The MAP Kinase immunoprecipitation is a set of reactions that detects MAP Kinase activity by its phosphorylation of myelin basic protein (**MBP**). The agarose beads are conjugated with an antibody of rabbit IgG anti MAP Kinase. This binds the MAP Kinase present in the cell extract. The MBP and ATP are then added to the mixture. The

natural action of the MAP Kinase in the presence of these two compounds is to take the phosphate from the ATP and phosphorylate the MBP (fig. 5). The phosphorylated-MBP is then electrophoretically separated from the other compounds, and detected with an anti phosphorylated-MBP antibody.

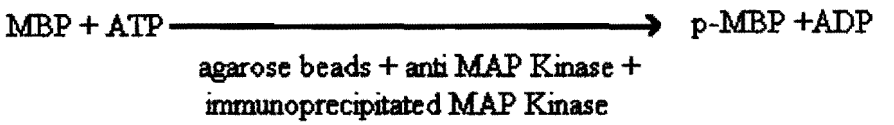

Fig. 5. MAP Kinase reaction. Immunoprecipitated MAP Kinase phosphorylates MBP in the presence of ATP.

For the MAP Kinase immunoprecipitation, the cells were handled in the same manner and exposed to the same conditions as the cells above. Three plates of cells were then reacted according to a MAPK Immunoprecipitation Assay Kit (7), which includes the immunoprecipitation of MAP Kinase, electrophoresis and immunoblotting. However, the third plate was left in the incubator until the end of the hour when Angiotensin II (1 micromolar) was added for five minutes. Angiotensin II is a known MAP Kinase inducer for vascular smooth muscle cells and was included as a positive control for MAP Kinase activity. The cells were lysed and the protein was extracted in the same manner as before. Once again the protein concentration was quantified with a Bradford protein assay and the samples were standardized to equal protein concentrations.

The cell extract was added to agarose beads that were bound to a MAP Kinase antibody. Induced cell extract was added to one tube that contained beads only, no anti MAP Kinase antibody. This provided a negative control, as no MAP Kinase could be precipitated without the antibody. After incubation and washes per the immunoprecipitation assay protocol (7), the kinase inhibitor, substrate and Mg^{++}/ATP cocktails were added. Again negative controls were in place as the myelin basic protein necessary to detect MAP Kinase activity was omitted in one tube and ATP, necessary for

the MAP Kinase activity was omitted in another. The tubes were sampled and an electrophoretic gel was run just as above. The protein used to detect MAP Kinase was then transferred to nitrocellulose paper and detected as above with both colorimetric and chemiluminescent detection methods.

Results

HSP90 antibody titration

The dot blot was performed to determine the optimal working concentration of the antibodies and which secondary detection method worked best. When using a 1/3000 dilution for the secondary antibody the colorimetric detection found the 1X and 1/5 dilution of cell extract, the 1/1000 antibody dilution detected the 1X and the 1/10 dilution of cell extract and the 1/500 antibody dilution was able to detect all three concentrations of the protein in the cell extract. The chemiluminescent detection found only the 1X concentration of cell extract in all three antibody concentrations. Thus we decided to use the colorimetric detection using a 1/500 antibody dilution.

Bradford protein assay

For each gel that was run, the concentration of protein in the samples was determined by a Bradford protein assay. The standards, which comprised the standard curve, showed optical densities ranging from 0.175 (1.80μg/200μl) to 0.014 (0.10μg/200μl). The unknown samples were diluted and the 1X, 1/10 and 1/100 dilutions were tested. Consistently, the 1/10 dilution resulted in an optical density, measured with a wavelength of 600 nm, within the range of the standard curve allowing for the protein concentration to be calculated accurately.

HSP90 detection

The results of two of the three successful experiments are shown in fig. 6. HSP90

levels were detectable in the control cells and did not increase in the hypoxic cells. The rainbow marker shows that we were detecting protein in the general area of 90kD; however the HSP90 protein standard was necessary to confidently say that we were detecting HSP90 in the samples of cell extract.

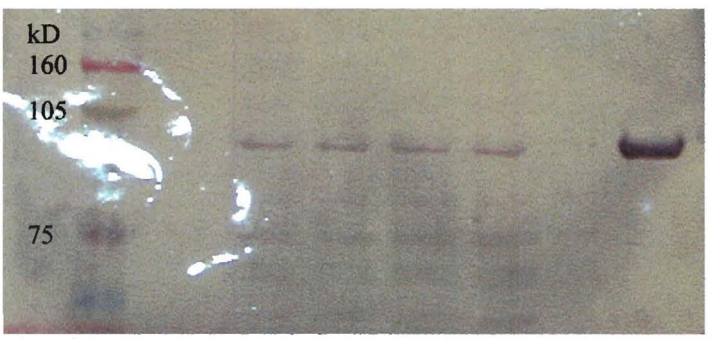

Fig. 6. Colorimetric detection. Rainbow marker on the left and HSP90 SPP770 standard on the right the four bands depict the HSP90 in four plates left to right, experiment 2: hypoxic, control, experiment 1: hypoxic, control.

MAP Kinase immunoprecipitation

Despite the results shown above the MAP Kinase immunoprecipitation was done. MAP Kinase activity is known to be at a certain level and we hypothesized that the level would be reduced by the hypoxia-induced increase in HSP90. In the one immunoprecipitation, no MAP Kinase activity was detected. Troubleshooting the immunoprecipitation cannot be done with certainty though because no activity was detected in the positive control.

Conclusions

The first objective of this research was to determine if the cultured smooth muscle cells react to hypoxia in the same manner as the excised rat aorta. Though the number of test was small (n = 3), we conclude that HSP90 production in the cultured cells was not induced when exposed to the same conditions that induced HSP90 production in the

excised rat aorta. There are two possible explanations for the difference in response to hypoxia between the cultured cells and the vascular tissue. The first is that the test conditions, which were sufficient to induce a hypoxic stress in the multi-layer smooth muscle of the aorta, were insufficient to stress the cultured cells. Though exposed to a lower partial pressure of oxygen than normal during the hypoxic time, a culture of cells is a monolayer and may be able to withstand the lower partial pressure of oxygen better than organized tissue. Another factor that may have influenced the stress of the cultured cells is the duration of the hypoxic condition. One hour may have been too short to induce a reaction. It is also possible that other components of the organized tissue, such as endothelial cells, are necessary to permit the induction of the HSP90 response.

Alternately, it is possible that the cultured smooth muscle cells do not respond to hypoxia like smooth muscle cells in freshly excised vascular tissue. This particular cell line is known to generate action potentials and demonstrate myosin kinase and creatine phosphokinase activity; however it is not clear whether the signal transduction cascade required for cell contraction is intact (6).

We note that the control samples from the cultured cells had a detectable level of HSP90, unlike the normoxia-control samples of freshly excised rat aorta (2). It is possible that the cultured cells normally express high levels of HSP90, which cannot be induced further by hypoxia. This could be because they are of embryonic origin, because the culture conditions themselves, although sufficient to maintain cell division, are a stressor, or because the property of the cells that permits them to be "immortalized" alters the stress response.

In spite of the inability to show an induction of HSP90 with hypoxia, we performed a MAP Kinase assay on control and hypoxic cells. We were unable to show that the cells contained MAP Kinase activity, as there was no detectable p-MBP. However, the results from the positive control were also negative, so it is possible that the negative results were due to technical difficulties. Additional experiments would be

necessary to determine whether the negative results were because of a lack of MAP Kinase activity in the cultured cell extract or because of improper technique.

In summary, at this point we must conclude that cultured A10 cells do not react in the same manner as excised rat aorta when exposed to one hour of moderate hypoxia. We had hypothesized that the hypoxia would induce HSP90, which would result in a reduced MAP Kinase activity. However, contrary to our expectations, the control cells expressed a high level of HSP90, which was not increased by the one hour of moderate hypoxia. We were therefore unable to confirm the hypothesis, based on these experiments in cultured A10 cells. However, different results may be obtained in excised vascular tissue, in which HSP90 has been shown to increase in response to moderate hypoxia (2).

References

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